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SHORT COMMUNICATION

High-resolution (mtDNA) melting analysis for simple and efficient characterization of Africanized honey bee *Apis mellifera* (Hymenoptera:Apidae)

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Abstract
Analysis of the mtDNA variation in *Apis mellifera* L. has allowed distinguishing subspecies and evolutionary lineages by means of different molecular methods; from RFLP, to PCR-RFLP and direct sequencing. Likewise, geometric morphometrics (GM) has been used to distinguish Africanized honey bees with a high degree of consistency with studies using molecular information. High-resolution fusion analysis (HRM) allows one to quickly identify sequence polymorphisms by comparing DNA melting curves in short amplicons generated by real-time PCR (qPCR). The objective of this work was to implement the HRM technique in the diagnosis of Africanization of colonies of *A. mellifera* from Argentina, using GM as a validation method. DNA was extracted from 60 A. mellifera colonies for mitotype identification. Samples were initially analyzed by HRM, through qPCRs of two regions (485 bp/385 bp) of the mitochondrial cytochrome b gene (cytb). This technique was then optimizing to amplify a smaller PCR product (207 bp) for the HRM diagnosis for the Africanization of colonies. Of the 60 colony samples analyzed, 41 were classified as colonies of European origin whereas 19 revealed African origin. All the samples classified by HRM were correctly validated by GM, demonstrating that this technique could be implemented for a rapid identification of African mitotypes in *Apis mellifera* samples.

Keywords *Apis mellifera* · Africanized honey bee · mtDNA characterization · High-resolution melting analysis · Geometric morphometrics

Introduction

The evolutionary history of *Apis mellifera* populations was modified over the centuries in relation to both natural and anthropic factors. Since the beginning of beekeeping 7.000 years ago, human activity has encouraged the transport of bees throughout the world, artificial selection, and an increase in gene flow between native and non-native subspecies (Harpur et al. 2012; Leclercq et al. 2018). Furthermore, accidental releases of non-native species has led to the expansion of hybridized strains of Africanized throughout the Americas (Kerr et al. 1982; Scott Schneider et al. 2004; Whitfield et al. 2006). After its arrival in Brazil in 1956, the African bee subspecies *Apis mellifera scutellata* began to spread throughout the American continent. The process of Africanization involved both maternal and paternal bidirectional gene flow between European and Africanized honeybees and is considered one of the most spectacular biological invasions yet documented (Pinto et al. 2005). Africanized bee populations express a reproductive, foraging and defensive behavior similar to *Apis mellifera scutellata*, and due to their high adaptability to tropical ecological conditions they rapidly expanded throughout the Americas (Sheppard et al. 1999; Diniz et al. 2003).

Although the increased defensiveness, high swarming rates, absconding and migration behavior of African bees were recognized as detriments to beekeeping, it was thought that selective breeding could modify these undesirable traits (Scott Schneider et al. 2004). Africanized (AHB)

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and European bees (EHB) show significant differences in a number of traits associated with foraging efficiency and foraging strategy. These differences give an advantage to EHBs over AHBs for honey production, and an advantage to AHBs for increased reproduction and colonization of new environments (Guzman-Novoa et al. 2020). Furthermore, it has been proved that the defensive behavior of honeybees can be affected by environmental conditions as well as by interactions between worker bees (Uribe-Rubio et al. 2003). Methods for a rapid discrimination of Africanized colonies provide therefore a great advantage for selective breeding.

Studies of feral populations suggest that Africanized honeybees expanded by maternal migration (Hall and Muralidharan 1989; Smith et al. 1989; Hall and Smith 1991). Variation in honey bee mitochondrial DNA (DNAmt) has allowed distinguishing evolutionary lineages using a variety of molecular methods, ranging from Restriction Fragment Length Polymorphisms (RFLP) to PCR-RFLP and direct sequencing Garnery et al. 1993; Franck et al. 2001; De La Rúa et al. 2005; Collet et al. 2006; Pinto et al. 2012; Meixner et al. 2013). Furthermore, morphological comparisons of wing venation patterns using automated measurement tools have previously been used to distinguish Africanized bees (Francoy et al. 2006, 2008; Baylac et al. 2008; Tofilski 2008; Miguel et al. 2011; Kandemir et al. 2011) with a high degree of agreement with molecular characterizations. However, these techniques are time-consuming and demand significant field expertise to complete the genetic characterization of bee colonies.

High-resolution melting (HRM) provides a low-cost, fast and sensitive alternative scanning method that allows the rapid detection of DNA sequence variants in a single step, which makes it appropriate for DNA sequence identification and genotyping (Vossen 2017; Erali et al. 2008; Reed et al. 2007; Santos et al. 2013; Marin et al. 2016). Melting curve analysis is a simple method to detect DNA sequence variations. The procedure consists in amplifying a target sequence using the polymerase chain reaction (qPCR) with a fluorescent double-stranded DNA (dsDNA) binding dye, melting of the fluorescent amplicons, and subsequent interpretation of melting curve profiles (Tucker and Huynh 2014). This method is highly sensitive for discriminating DNA sequence variants and has been previously used to identify the genetic origin of *Apis mellifera* honey samples (Soares et al. 2019). Here, we present a simple and efficient technique for the diagnosis of Africanized *A. mellifera* colonies using High-Resolution Melting (HRM), using the wing geometric morphometric approach as complementary validation of our molecular (DNA_{MT}) characterizations.

Materials & methods

Sample collection

A total of 60 samples from managed honey bee colonies were collected from 16 Argentine provinces during 2016–2018 (see Supplementary Materials for sampling locations). Samples were preserved in 96 % ethanol and remitted to the C.I.A.S. (Centro de Investigación en Abejas Sociales, Universidad Nacional de Mar del Plata) for further analysis.

Geometric morphometric characterization

For each colony sampled, the left forewing of 10 workers, were dissected (Tofilski 2008), mounted in glass photographic frames and scanned with a Plustek opticfilm 8100 (7200 dpi). For every wing image, the coordinates of 19 homologous landmarks (Francoy et al. 2008) were manually plotted at the wing vein (Fig. 1) using the software tpsDIG, v2.16 and tpsUtil v1.4 (Rohlf 2010). Additionally, wing images of 50 different colonies representing the subspecies *A.m.scutellata* (Morphometric Bee Data Bank in Oberursel, Germany) were included in the analysis as a non-hybridized reference group. Landmark coordinates were imported into the MORPHOJ software package (Klingenberg 2011). Alignments were performed using Procrustes fit (translation, proportion, and rotation) (Dryden and Mardia 2016). Generalized adjustment of the Procrustes method was performed following Viscosi and Cardini (2011). Based on the spectral decomposition of covariance, Principal Component Analysis (PCA) and Canonical Variate Analysis (CVA) were performed with all measurements. Mahalanobis distances were calculated (Klingenberg and Monteiro 2005) allowing to assess variation from each colony in relation to the *A. m. scutellata* reference sample. Discriminant function analysis (DFA), with cross-validation and permutation tests (Procrustes distance and the T-square statistic) was used to analyze the separation between groups of colonies (i.e., Africanized vs. Non Africanized).

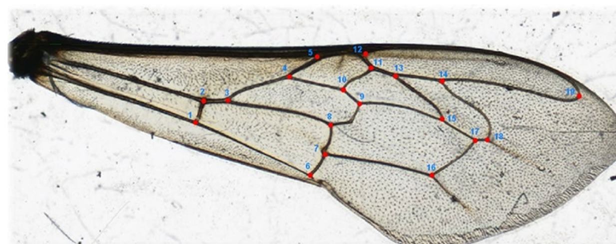


Fig. 1 Location of 19 wing landmarks used to characterize honeybee subspecies by geometric morphometric variation of vein junctions

Mitotype characterization

For molecular mitotype identification, total genomic DNA was extracted from 60 bee thoraces (1 bee/colony) using the High Pure PCR Template Preparation kit (Roche Diagnostics). The samples were subjected to two independent real-time polymerase chain reactions (qPCR). On the one hand, a region of the mitochondrial gene of cytochrome b (cyt b) was amplified using the Apis-F (5′-TATGTACTACCATGA GGA-CAAATATC-3′) and Apis-R (5′-ATTA-CACCTCCTA ATTTATTAGGAAT-3′) primers (Crozier et al. 1991), which generate a control amplicon of 485 bp (Melting temperature Tm = 80 ± 0.2° C) for European and African bees. In a second amplification reaction, the combination of primer Apis-R with a new primer AHB-F (5′-CATTACTCTGAG GTGGGTTC-3′) (Szalanski and McKern 2007) was used to generate an amplicon of 385 bp (Tm: 79 ± 0.2° C) unique to the African mitotype. This second amplification allowed sequence specificity analysis of amples using the High-Resolution Melting technique (High Resolution Melting, HRM). To corroborate size and amplification specificity, PCR products were resolved on agarose gel electrophoresis and purified using the Accuprep gel purification kit (Bioneer, South Korea). In all cases, positive controls were used for both European (EHB) and Africanized (AHB) bees to corroborate product sizes and melting curves (Fig. 2). Additionally, partial sequences of the cytochrome b mitochondrial gene were obtained and a phylogenetic tree was constructed using MEGA7 (Kumar et al. 2016) to compare positive controls with previously reported sequences from Genebank (accession numbers: EF016643-48). The F81+i model was identified by jmodeltest2 (Darriba et al. 2012) as the best fitting substitution model as determined by the lowest AICc. ML trees were obtained using a heuristic search method (10,000 random addition replicates tree-bisection-reconnection, TBR, branch swapping) and bootstrap analyses with 1000 replications (Supplementary Material 1).

A new reverse primer AFR207R (5′-AGGCAAATAAAT GAAGA-3′) was designed to amplify a smaller product (207 bp) in combination with the Apis-F primer to determine Africanization by HRM analysis. PCR amplifications were performed in a 20 µl total volume containing 10 µl of 2X MyTaq PCR mix (Bioline, London, UK) master mix, 1 µM of each primer, and 1 µl (5 ng µl^{-1})of DNA template. The PCR cycling profile for the Apis-F/Apis-R primers consisted of an initial denaturation of 2 min at 95° C, and 40 cycles of 94 °C 20 s, 52 °C 30 s, 72 °C 30 s. The PCR cycling profile for the AHB-F/Apis-R primers included an initial denaturation of 2 min at 95° C, and 40 cycles of 94 °C 20 s, 50 °C 30 s, 72 °C 30 s. After amplification, melting curve analyses were performed to confirm specific amplification of PCR products. All qPCR reactions were carried out in a thermocycler Rotor-Gene (Qiagen, Hilden, Germany) in a final volume of 20 µl using EvaGreen as fluorescent intercalating dye (KAPA Fast, Biosystems, Woburn, USA). The cycling program for Apis-F/AFR207R consisted of an initial denaturation of 2 min at 95° C, and 40 cycles of 94 °C 20 s, 48 °C 20 s, 72 °C 20 s. HRM was carried out immediately

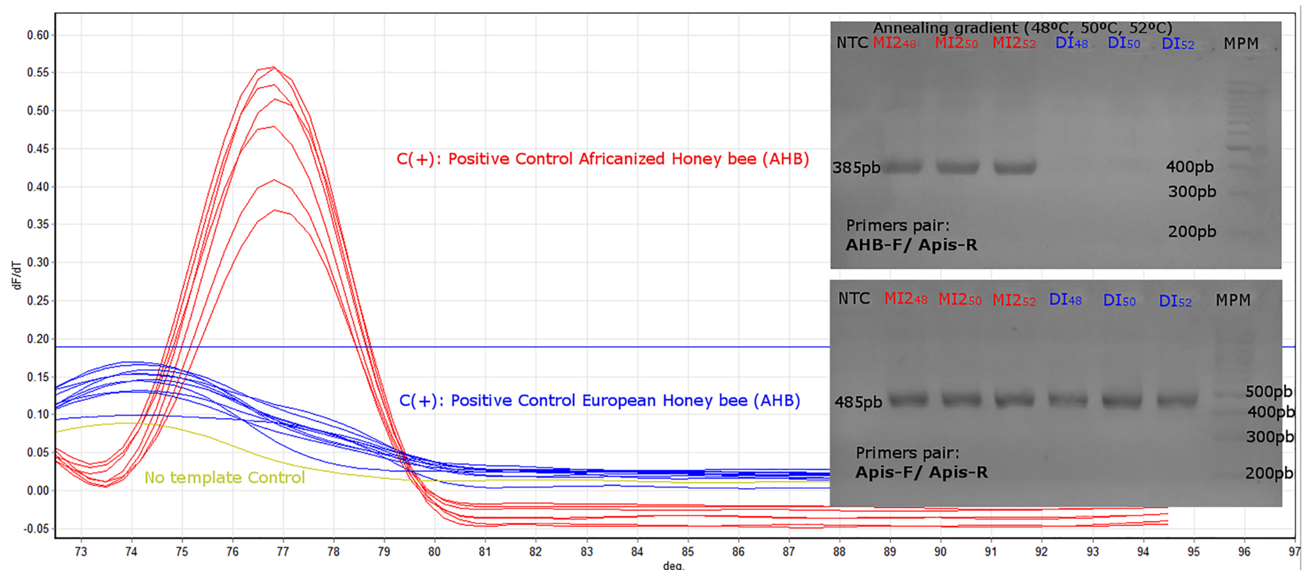
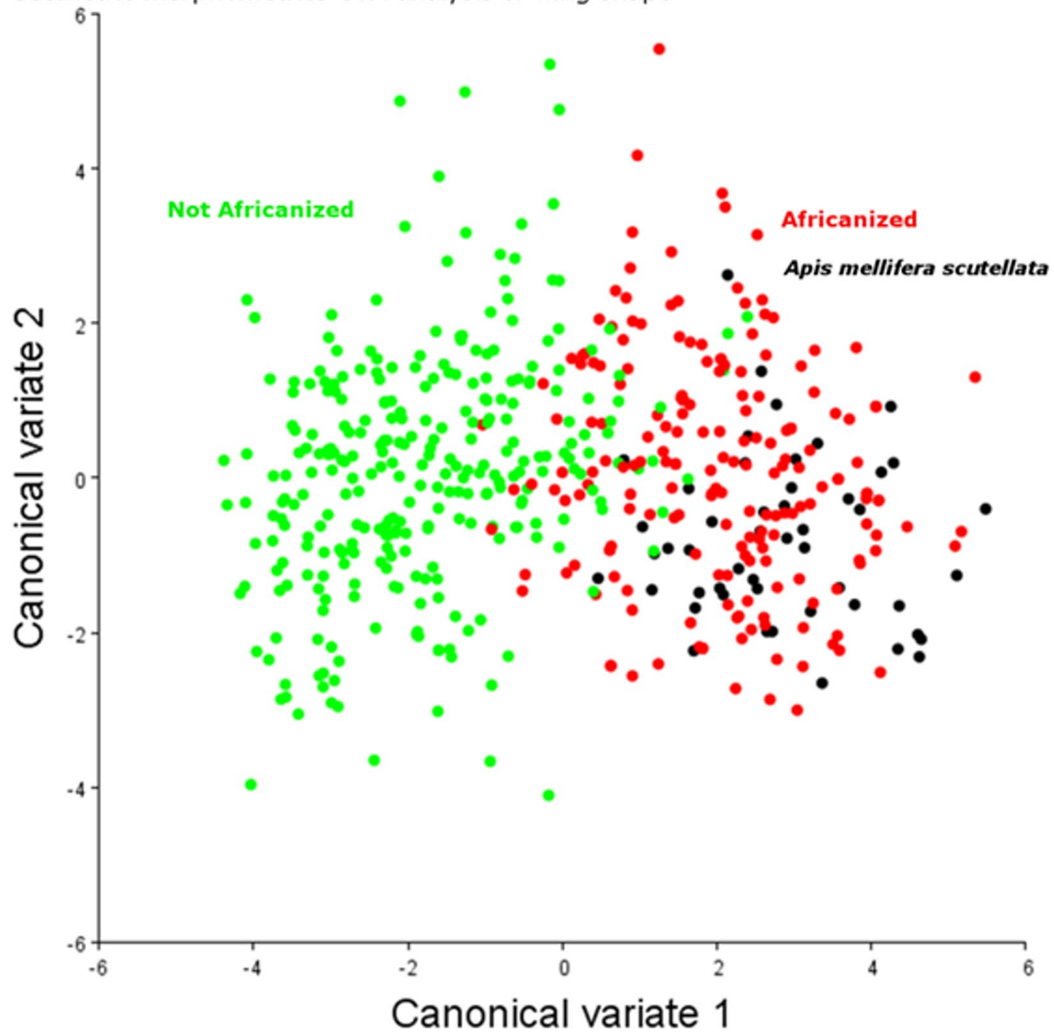


Fig. 2 Real-time polymerase chain reactions (qPCR) carried out to validate modifications of the methodology previously used by Szalanski and McKern (2007). Positive controls were used for both European (EHB) and Africanized (AHB) bees to corroborate PCR product sizes and melting curves. The vertical axis represents the rate of change in relative fluorescence units (RFU) with time (T) (-d (RFU)/dT) versus temperature (°C) on the horizontal axis. On the right margin, cytochrome b mitochondrial gene amplification products with annealing gradient (48°, 50°, 52°) are visualized by agarose gel electrophoresis


A Geometric morphometrics CVA analysis of wing shape
Not Africanized
Africanized
Apis mellifera scutellata
Canonical variate 2
Canonical variate 1
6
4
2
0
-2
-4
-6
-6
-4
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2
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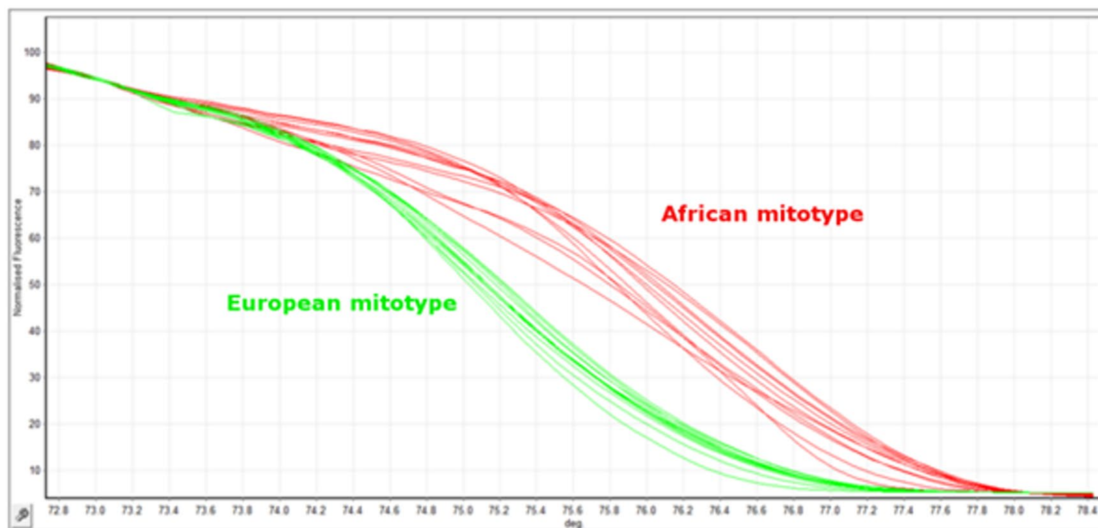
B High-Resolution Melting (HRM) mitotype characterization
African mitotype
European mitotype
Normalised Fluorescence
deg

◂**Fig. 3** **A** Geometric morphometric analysis of wing shape. Canonical variate analysis (CVA) scatter plot (CV1 vs. CV2) from tree different groups of honeybee colonies: Non Africanized (green dots), Africanized (red dots), and reference samples of *Apis mellifera scutellata* (from morphometric bee data bank in Oberursel, Germany) (black dots). **B** High-Resolution Melting (HRM). Melting curve analysis used to differentiate honeybee mitotype by normalized fluorescence curves. Fluorescence is represented on the vertical axis and temperature on the horizontal axis. Fluorescence curves of colonies with African mitotype are shown in red whereas those with European mitotype are shown in green

after PCR at temperature increment steps of 0.1 °C from 75 °C to 95 °C. The results were analyzed with Rotor Gene Q software, version 1.7.94. A genotype identification confidence cut off value of 90 % was set up in the genotyping module of the software. PCR reactions of each genotype were performed in triplicate.

After HRM analysis, some PCR products were run on agarose gels and purified using Accuprep Gel Purification Kit (Bioneer, South Korea). Purified fragments were then sequenced using BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) with the ABI Prism 310 Genetic Analyzer (Applied Biosystems) to confirm mitochondrial haplotypes.

Results & discussion

Geometric morphometric characterization

The canonical variation analysis (CVA) performed on the covariance matrix of wing morphometric traits revealed that the first component explained a cumulative variation greater than 85 % for comparison groups (see Supplemental Materials 2). The patterns of wings variation found in Africanized colonies were similar to the *A. m. scutellata* reference group (Fig. 3). Mahalanobis distances of the reference to Africanized colonies ($\mu = 4.07$; SD ± 0.51) were significantly smaller ($t_{58} = 16.52$, $p = 3.72$ E-24) than to European colonies ($\mu = 9.20$; SD ± 1.24) (Table 1). Discriminant functions analysis allowed to compare and significantly differentiate both groups of observations: Africanized vs. European (MD = 6.0776; T-square: 508.6447, P-value (parametric): < 0.0001) (Supplementary Material 2).

Mitotype characterization

Of 60 samples analyzed, 41 were classified of European origin and 19 of African origin (Table 1). In addition, 15 samples of both African and European origin were purified and sequenced. When the sequences were aligned and compared, different polymorphisms were found in samples classified as European that were collected at different latitudes (Supplementary Materials 3). All samples classified by HRM were correctly validated by the morphometric approach (Fig. 3), demonstrating that this technique could be implemented for rapid identification of the African mitotype in *Apis mellifera* samples.

PCR based assays for the amplification of mitochondrial DNA have been previously used in studies of the Africanization process in neotropical feral and managed honey bee populations. The cytochrome b/BglII assay, first developed by Crozier et al. (1991) and further validated by Pinto et al. (2003), is a simple and relatively inexpensive test suited for screening large numbers of samples and easily identifiable mitotypes. However, since mtDNA provides information only about maternal ancestry, this marker should be used in conjunction with morphometric analyses (Nielsen et al. 1999). Simple and rapid identification of samples from Africanized *Apis mellifera* populations provide an important methodological advantage, particularly when a large number of samples need to be analyzed in a short period of time.

Complementing both morphometric and molecular techniques can optimize characterization and improve genetic selection in increased dynamic populations. Although it is very complex to determine the degree of Africanization, especially in transition zones, where a mixture of subspecies occurs, this method could be very useful for a preliminary characterization of Africanized honeybee populations. Melting curve analysis is a highly sensitive method that can be carried out in a single amplification reaction, reducing costs and sampling efforts. In the present work all the samples classified by HRM were correctly validated by GM, demonstrating that this technique can be implemented for a rapid identification of the African mitotype in *Apis mellifera* samples from natural populations.

Table 1 The sampled bee colonies are shown geo-referenced, indicating the origin (country / province) and the result obtained for both analysis methods (High-Resolution Melting mitotype characterization / Geometric morphometric analysis of wing shape). In addition, those that were sequenced are indicated with an asterisk (*)

Sample: Country/ Province Sequenced samples*	Latitude	Longitude	High-Resolution Melting mitotype characterization (Apis F-AFR207R)	Geometric morphometric analysis of wing shape (Malhanobis distance to *A.m.scutellata*)
Venezuela/LARA			African	3.3021
Venezuela/LARA			African	3.3519
Venezuela/LARA			African	3.9132
ARG/MISIONES	26°55′59.99″S	55° 3′59.98″O	African	4.5237
ARG/MISIONES	27°14′39.11″S	55°32′23.43″O	African	2.7803
ARG/MISIONES*	27°23′1.56″S	54°44′26.23″O	African	4.0756
ARG/MISIONES*	27°35′7.51″S	55° 8′6.61″O	African	3.8263
ARG/CORRIENTES	27°22′17.45″S	58°30′39.47″O	African	4.6582
ARG/CORRIENTES	29°10′22.52″S	56°39′12.94″O	African	4.5463
ARG/CORRIENTES	27°30′40.72″S	58°33′24.24″O	African	4.2228
ARG/CORRIENTES	29°11′4.55″S	58° 4′24.84″O	African	5.0786
ARG/JUJUY	24° 7′17.20″S	65°24′6.28″O	African	3.4094
ARG/JUJUY	24°22′13.96″S	65°19′54.60″O	African	4.2896
ARG/SALTA	24°47′19.57″S	65°24′44.80″O	African	5.7171
ARG/JUJUY	24°11′8.83″S	65°17′58.12″O	African	3.4094
ARG/CATAMARCA*	28°44′38.52″S	65°57′3.09″O	African	4.8180
ARG/SANTA FE	29° 8′46.92″S	59°38′35.50″O	African	3.9162
ARG/CHACO	26°32′22.88″S	59°20′31.10″O	African	3.8442
ARG/FORMOSA	25° 6′55.96″S	58°15′25.47″O	African	3.6909
ARG/LA RIOJA	31°30′2.60″S	66°42′46.42″O	European	6.7251
ARG/RIO NEGRO	40° 6′8.47″S	64°27′29.62″O	European	8.0565
ARG/CHUBUT	42° 2′54.07″S	71°30′56.04″O	European	9.2661
ARG/STA. CRUZ*	46°32′55.06″S	71°37′55.12″O	European	10.0491
ARG/SGO. ESTERO	27°46′33.85″S	64°14′17.64″O	European	8.1421
ARG/CORDOBA*	33° 9′26.61″S	64°21′6.95″O	European	10.0758
ARG/BS.AS.*	38°10′7.97″S	57°38′8.63″O	European	11.0839
ARG/BS.AS.*	38°43′5.94″S	62°15′58.85″O	European	8.0354
ARG/BS.AS.	38°33′1.48″S	58°41′47.08″O	European	7.9023
ARG/BS.AS.	38°12′18.48″S	61°46′10.98″O	European	7.6783
ARG/BS.AS.	38° 1′1.30″S	57°40′0.72″O	European	8.1564
ARG/BS.AS.	38° 9′48.39″S	58°46′54.07″O	European	8.1902
ARG/BS.AS.	37°50′47.22″S	58°15′19.36″O	European	7.5908
ARG/BS.AS.	38°33′1.48″S	58°41′47.08″O	European	8.3012
ARG/BS.AS.	37°46′7.37″S	57°41′47.29″O	European	7.6030
ARG/MENDOZA	34°37′59.06″S	68°20′25.11″O	European	10.4246
ARG/MENDOZA	34°37′59.06″S	68°20′25.11″O	European	9.9511
ARG/MENDOZA	34°37′59.06″S	68°20′25.11″O	European	9.1564
ARG/MENDOZA	34°37′59.06″S	68°20′25.11″O	European	8.0439
ARG/MENDOZA	34°37′59.06″S	68°20′25.11″O	European	10.3566
ARG/MENDOZA*	34°37′59.06″S	68°20′25.11″O	European	10.5666
ARG/MENDOZA	34°37′59.06″S	68°20′25.11″O	European	10.6392
ARG/MENDOZA	34°37′59.06″S	68°20′25.11″O	European	10.9457
ARG/MENDOZA	34°37′59.06″S	68°20′25.11″O	European	9.5576
ARG/MENDOZA	34°37′59.06″S	68°20′25.11″O	European	7.7008
ARG/RIO NEGRO	40° 6′8.47″S	64°27′29.62″O	European	8.0565
ARG/CORRIENTES	30°15′31.42″S	57°38′9.38″O	European	8.4099

Table 1 (continued)

Sample: Country/ Province Sequenced samples*	Latitude	Longitude	High-Resolution Melting mitotype characterization (Apis F-AFR207R)	Geometric morphometric analysis of wing shape (Malhanobis distance to *A.m.scutellata*)
ARG/CORRIENTES*	30°15′31.42″S	57°38′9.38″O	European	8.4334
ARG/BS.AS.*	38°10′7.97″S	57°38′8.63″O	European	11.9532
ARG/BS.AS.	38°10′7.97″S	57°38′8.63″O	European	10.3487
ARG/BS.AS.	38°10′7.97″S	57°38′8.63″O	European	11.0590
ARG/BS.AS.	38°10′7.97″S	57°38′8.63″O	European	9.0189
ARG/BS.AS.*	38°43′5.94″S	62°15′58.85″O	European	8.8409
ARG/BS.AS.	38°12′18.48″S	61°46′10.98″O	European	9.5678
ARG/BS.AS.	38°12′18.48″S	61°46′10.98″O	European	8.9733
ARG/BS.AS.	38°12′18.48″S	61°46″10.98″O	European	9.8491
ARG/BS.AS.*	38°12′18.48″S	61°46′10.98″O	European	9.6569
ARG/BS.AS.	38° 5′5.98″S	63°25′50.79″O	European	11.0655
ARG/BS.AS*	38° 5′5.98″S	63°25′50.79″O	European	9.0876
ARG/BS.AS*	38° 5′5.98″S	63°25′50.79″O	European	8.6547
ARG/LA PAMPA*	38° 5′5.98″S	63°25′50.79″O	European	10.2653

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